Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 11259–11263, December 1992
Pharmacology

Basal release of nitric oxide from aortic rings is greater in female rabbits than in male rabbits: Implications for atherosclerosis

(endothelium-derived relaxing factor/superoxide dismutase/L-arginine/gender difference/estrogen)

TOSHIO HAYASHI*†, JON M. FUKUTO*, LOUIS J. IGNARRO*, AND GAUTAM CHAUDHURI*‡§

Departments of ‡Obstetrics and Gynecology and *Pharmacology, University of California, Los Angeles, School of Medicine, Los Angeles, CA 90024

Communicated by Charles H. Sawyer, September 1, 1992 (received for review August 1, 1992)

ABSTRACT **Estradiol is known to exert a protective effect against the development of atherosclerosis, but the mechanism of this hormonal action is unknown. One of the early events in the development of atherosclerosis is the adhesion of macrophages to endothelial cells, and nitric oxide (NO) inhibits this process. We show that basal release of NO is greater with endothelium-intact aortic rings from female rabbits than those from males. Oophorectomy diminishes both circulating estradiol concentration and basal release of NO to levels seen in male rabbits. These data establish that basal NO release from endothelium-intact aortic rings depends on circulating estradiol concentration and offer an explanation for the protective effect of estradiol against the development of atherosclerosis.**

Women in the reproductive age group are protected from coronary artery disease when compared with men (1), although the exact mechanism by which this is achieved is unknown. One of the early events in the development of atherosclerosis is the adhesion of macrophages to endothelial cells (2), and substances produced by the normally functioning endothelium probably are critical in preventing this occurrence. An important substance produced by the endothelium, initially called endothelium-derived relaxing factor (3) and more recently proposed to be nitric oxide (NO) (4, 5), mediates the vascular relaxation produced by various endothelium-dependent vasodilators, such as acetylcholine (ACh), bradykinin, and the calcium ionophore A-23187 (3, 6, 7). NO also prevents adhesion of monocytes (8) and platelets (9) to endothelial cells.

We hypothesized that the mechanism by which 17β-estradiol (E_2) inhibits the development of atherosclerosis in the female mammal is the increased formation of NO by endothelial cells that probably modulate the early events in the development of atherosclerosis. We therefore decided to investigate the release and actions of NO from aortic rings obtained from male, female, and oophorectomized rabbits. Rabbits were chosen as the animal model because the unmated female of this species remains in persistent estrus, and these animals are thereby under the constant influence of E_2 without any significant compounding influence of progesterone. Aortic rings were selected for study because feeding a high-cholesterol diet to rabbits leads to atherosclerotic lesions that are prominent in the thoracic aorta (10).

MATERIALS AND METHODS

Reagents. Acetylcholine chloride (ACh), phenylephrine hydrochloride, A-23187, L- and D-arginine hydrochloride, hemoglobin, indomethacin, potassium chloride, and N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME) were obtained from Sigma. Copper–zinc superoxide dismutase (SOD; 4300 units/mg of protein) was obtained from DDI Pharmaceuticals (Mountain View, CA). Nitroglycerin [NG; 10% (wt/wt) triturate in lactose] was from Imperial Chemical Industries. N^G-Methyl-L-arginine acetate (L-NMA) was synthesized and purified by the method of Patthy *et al.* (11). Oxyhemoglobin solutions were prepared as described (12). Krebs bicarbonate solution, pH 7.4, contained 118 mM NaCl, 4.7 mM KCl, 1.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 11 mM glucose, and 0.002 mM disodium ethylenediaminetetraacetic acid and was gassed with 95% O_2/5% CO_2. Depolarizing KCl solution had a composition similar to Krebs buffer, except that NaCl was replaced by an equimolar amount of KCl. All concentrations indicated in the *in vitro* studies are expressed as final bath concentrations.

Animals. New Zealand White rabbits weighing between 3 and 3.5 kg were used. Castration was done under anesthesia (ketamine at 66 mg/kg and xylazine at 6 mg/kg i.m.).

Preparation of Rabbit Aortic Rings and Tension Recording. The preparation of rabbit aortic rings was similar to that described by Furchgott and Zawadzki (3). Briefly, the rabbits were sacrificed by exsanguination after anesthesia with pentobarbital (50 mg/kg i.v.). The thoracic aortas were carefully removed to protect the endothelial lining, cleared of adhering fat and connective tissues, and cut into 3-mm-wide transverse rings with the aid of a Stoelting model 51425 tissue chopper (Stoelting). The rings were mounted under 1 g of resting tension on stainless-steel hooks in 25-ml-capacity muscle chambers and were bathed in Kreb's bicarbonate solution, pH 7.4 at 37°C. Tension was measured isometrically using force displacement (FT 03C) transducers and was displayed either on a Grass polygraph (model 79D) or on a modified Sony recorder. In studies to elucidate the tone-related release of NO from endothelium-intact aortic rings, moderate vascular tone (35–50% of the contractile response from 122 mM KCl) was induced with low concentrations of phenylephrine (10^{-7} M to 2×10^{-7} M). In one series of experiments, concentration-related relaxant responses to SOD (0.1–100 units/ml) were obtained, and at the peak of vasorelaxation, oxyhemoglobin (1 μM) was used to inactivate NO. In some experiments, in addition to SOD (100 units/ml), catalase (200 units/ml) was also present to rule out the role of peroxide in this process. In another series of experiments, concentration-related contractile responses to L-NAME and L-NMA (1–100 μM) were assessed by using separate rings.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: SOD, superoxide dismutase; L-NMA, N^G-methyl-L-arginine acetate; L-NAME, N^G-nitro-L-arginine methyl ester hydrochloride; E_1, estrone; E_2, 17β-estradiol; NG, nitroglycerin; ACh, acetylcholine.

†Present address: Department of Geriatrics, Nagoya University School of Medicine, 65 Tsuruma-cho, Showa-ku, Nagoya-466, Japan.
§To whom reprint requests should be addressed at: Departments of Obstetrics and Gynecology and Pharmacology, University of California, Los Angeles, School of Medicine, 10833 LeConte Avenue, Los Angeles, CA 90024-1740.

Experiments were also undertaken to elucidate gender differences, if any, on the responsiveness of endothelium-intact aortic rings to endothelium-dependent vasodilators, ACh, and calcium ionophore A-23187 and on the responsiveness of endothelium-denuded aortic rings to endothelium-independent vasodilator NG. In these experiments, submaximal tension was initially induced with phenylephrine (3–3.5 $\times 10^{-7}$ M), after which cumulative concentration responses were obtained with ACh, A-23187, and NG. Some experiments were done after indomethacin (5×10^{-6} M) was added to muscle chambers and allowed to incubate for 60 min before the start of the experiments to rule out the contribution of prostanoids.

Amino Acid Assays. *Plasma.* The plasma arginine and citrulline were assayed by described methods (13, 14). Two aliquots (200 μl) were diluted with 2000 μl of methanol. The samples were kept for 10 min to facilitate deproteinization and then centrifuged at 10,000 × *g* for 15 min. After filtration through a 0.45-μm filter, an aliquot (700 μl) was mixed with 700 μl of *o*-phthalaldehyde reagent (fluoraldehyde reagent, Pierce) for 1 min at room temperature, and 20-μl samples were then applied to a 100 × 4.6 (i.d.)-mm Microsorb AAAnalysis, type O HPLC column fitted with a 15 × 4.6 (i.d.)-mm guard column (Rainin Instruments). The column was isocratically eluted at room temperature with solvent: 10 mM KH_2PO_4, pH 5.9/acetonitrile/methanol/tetrahydrofuran, 76.5:11.5:11.5:1 (vol/vol). Flow rate was adjusted to 0.8 ml/min, and fluorescence of the eluent was continuously monitored with a fluorescence detector, set to an excitation wavelength of 338 nm and an emission wavelength of 425 nm. Endogenous amino acids were identified by comparison with authentic standards. The concentrations of the amino acids were calculated by using standard calibration curves for individual amino acids. In each experiment, separate standard curves for authentic L-arginine and L-citrulline were constructed by using a range of arginine and citrulline concentrations that bracketed those found in the tissue samples. The detection limit was ≈0.025 pmol per amino acid. The calibration curves obtained were linear in a range of 1–250 pmol.

Aortic rings. The arginine and citrulline concentrations of aortic rings were estimated by using described methods (15, 16). Three to four aortic rings (3-mm width weighing ≈15–25 mg per ring) were dissected out from each rabbit and cleaned of loose connective tissue in Krebs bicarbonate solution. The rings were then homogenized in 1 ml of 6% (vol/vol) trichloroacetic acid (ground-glass tissue grinder) and allowed to settle for 120 min, after which the homogenate was centrifuged at 3000 × *g* for 15 min at 4°C. The supernatant was extracted and adjusted to pH 7.0 and then filtered. An aliquot (700 μl) of the filtered supernatant was mixed with 700 μl of *o*-phthalaldehyde reagent for 1 min at room temperature and then assayed by HPLC, as described for estimation of plasma arginine and citrulline.

Radioimmunoassay for Steroids. The plasma concentrations of E_2 and estrone (E_1) were measured by specific RIAs, as described (17). Briefly, 0.6–0.8 ml of plasma (pooled) with added known amounts (≈400 cpm) of [^{3}H]E_2 and [^{3}H]E_1 (15, 16) was extracted with 7 ml of fresh diethyl ether, and the extract was evaporated to dryness. The dried plasma extract was dissolved in isooctane and then applied onto a Celite column for chromatographic separation of E_1 and E_2, as described by Brenner *et al.* (18). Steroid fractions collected from the column were dried and reconstituted with assay buffer for RIA. The results showed that peaks of radioactive and immunoreactive estrogens coincided in Celite column fractions (17).

Assay for Lipids. Total cholesterol and triacylglycerols were measured by enzymatic techniques with an ABA-200 bichromatic analyzer (Abbott), high-performance cholesterol reagent (N 236691, Boehringer Mannheim Diagnostics) and triacylglycerol agent (N 6097, Abbott) (19). Low density lipid and high density lipid cholesterols were measured according to the standardized procedures of the Lipid Research Clinic Manual (20); the assays were done in the Lipid Research Laboratory, University of California, San Diego, by J. Juliano. This laboratory is under the continuous standardization program of the Centers for Disease Control, Atlanta, GA.

Table 1. Mean serum concentrations of total cholesterol, high density lipoprotein cholesterol, and triacylglycerols in male and female rabbits

	Lipid profile, mg/dl		
	Total cholesterol	HDL cholesterol	Triacylglycerol
Males	57.9 + 15.6	27.4 + 3.2	82.4 + 13.7
Females	52.9 + 6.0	28.3 + 3.5	98.5 + 25.3

Each value represents a mean (±SEM) from six to eight animals; no significant differences in values were seen between the two groups. HDL, high density lipoprotein.

Histological Examination. The aortic rings used for *in vitro* experiments on tension recording were examined histologically by hematoxylin/eosin staining to confirm the presence or absence of endothelium and also assessed for lipid disposition by using Sudan red stain.

Calculations and Statistical Analysis. Relaxation was measured as the percentage of decrease in tension below the evaluated tension elicited by precontracting arterial rings with phenylephrine. Contraction was measured as the percentage of increase in tension above the evaluated tension elicited by precontracting arterial rings with phenylephrine. Values are expressed as means ± SEMs and represent unpaired data. Comparisons of means were made by using the Student *t* test for unpaired values; when more than two means were compared, an analysis of variance with repeated measurements was used. If a significant F value was found, Scheffe's test for multiple comparisons was used to identify differences among groups. Values were considered significant when P was < 0.05.

RESULTS

Serum Lipids and Plasma Steroid Concentration. The serum lipids showed no differences between male and female rabbits (Table 1). Plasma E_1 and E_2 concentrations were higher in female rabbits when compared with males. Six weeks after oophorectomy, the E_1 and E_2 concentrations were significantly lower when compared with ovary-intact female rabbits, and the values corresponded to those seen in male

Table 2. Mean plasma concentrations of E_1 and E_2 in male rabbits, female rabbits, and oophorectomized female rabbits (6 weeks after oophorectomy)

	Profile of plasma estrogens, pg/ml	
	E_1	E_2
Males	11.91 + 2.11*	14.14 + 2.65*
Females	21.68 + 2.88	26.58 + 3.28
Oophorectomized females	12.37 + 2.26*	12.24 + 4.66*

Each value (± SEM) represents a mean from six to eight animals. E_1 and E_2 concentrations were significantly lower in males than in female rabbits. Six weeks after oophorectomy values were significantly lower than in ovary-intact females, and these values corresponded to those seen in male rabbits.

*Value is significantly less ($P < 0.05$) compared with that in normal female rabbits.

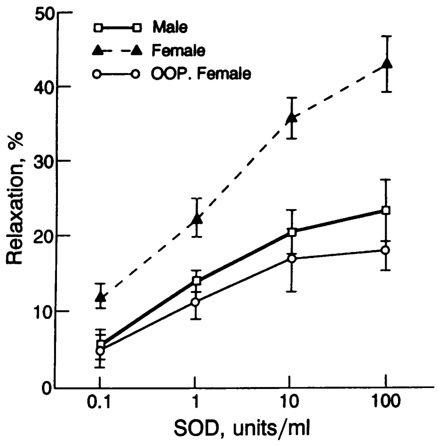

FIG. 1. Mean ± SEM percent relaxation of endothelium intact aortic rings to different concentrations of SOD. Aortic rings were moderately contracted (30–50% of that produced by 122 mM KCl) by phenylephrine before obtaining cumulative responses to SOD. Relaxation was significantly greater in aortic rings from female rabbits than in aortic rings from either male or oophorectomized (OOP) female rabbits.

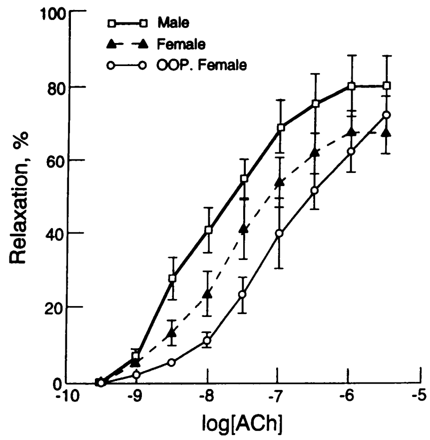

FIG. 3. Mean ± SEM percent relaxation of endothelium intact aortic rings from male, female, and oophorectomized (OOP) rabbits to different ACh concentrations. Aortic rings were submaximally contracted with phenylephrine before obtaining cumulative responses to ACh; responses were similar in all three groups.

rabbits. The plasma E_1 and E_2 values in the different groups are shown in Table 2.

Tone-Related Release of NO. In studies designed to assess the tone-related release of NO from phenylephrine-precontracted aortic rings, SOD produced concentration-related relaxant responses, and the magnitude of relaxant responses was greatest in endothelium-intact aortic rings from ovary-intact female rabbits. The magnitudes of relaxant responses of aortic rings obtained from oophorectomized females to SOD resembled those seen with aortic rings from males (Fig. 1). Addition of catalase (data not shown) did not produce any additional changes from those seen with SOD alone. At the peak of the relaxant response from SOD, addition of 1 μM oxyhemoglobin, a known inactivator of NO, caused contraction (data not shown).

In separate experiments, after moderate tone was induced with phenylephrine, we found that both L-NMA and L-NAME elicited vasoconstriction in a concentration- and endothelium-dependent manner; only the results obtained with L-NMA are shown (Fig. 2). The magnitude of vasoconstriction produced by L-NMA was greater in aortic rings from ovary-intact female rabbits than in those obtained from males. In contrast, the magnitude of those contractions was similar in aortic rings from males and oophorectomized rabbits (Fig. 2). Control aortic rings that were devoid of any tone or moderately precontracted endothelium-denuded aortic rings did not contract in response to L-NMA or L-NAME and did not relax in response to SOD (data not shown). These findings confirmed that the presence of endothelium and active vasomotor tone was necessary for tone-related NO release.

Relaxation Responses to ACh, A-23187, and NG. ACh (Fig. 3) and A-23187 (data not shown) produced concentration-dependent relaxation of endothelium-intact aortic rings precontracted with phenylephrine. NG relaxed precontracted endothelium-denuded aortic rings (Fig. 4). No significant differences in the relaxant responses to these vasodilators were seen in aortic rings from male, ovary-intact female, or oophorectomized female rabbits (Figs. 3 and 4). The responsiveness of the aortic rings was not modified after preincubation with indomethacin (data not shown).

Plasma and Tissue Arginine and Citrulline Concentrations. No significant differences in the plasma concentrations of arginine (males, 56.62 ± 12.10 μM; females, 79.76 ± 12.06 μM; and oophorectomized females, 89.86 ± 20.86 μM) or citrulline (males, 27.30 ± 5.81 μM; females, 40.20 ± 7.24 μM; and oophorectomized females, 45.14 ± 13.20 μM) were observed between male and female rabbits. Similarly, in

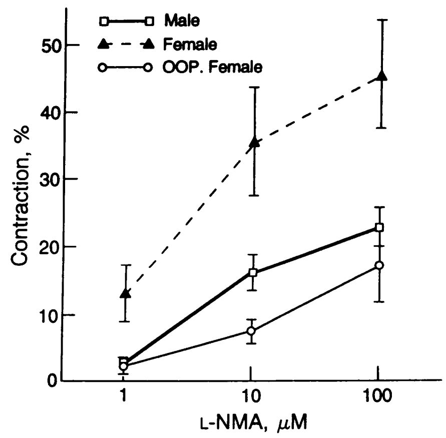

FIG. 2. Mean ± SEM percent contraction of endothelium intact aortic rings to different concentrations of L-NMA. The aortic rings were moderately contracted, as described for Fig. 1, before obtaining cumulative responses to L-NMA. The magnitude of contraction was significantly greater in aortic rings from female rabbits than in aortic rings from either male or oophorectomized (OOP) female rabbits.

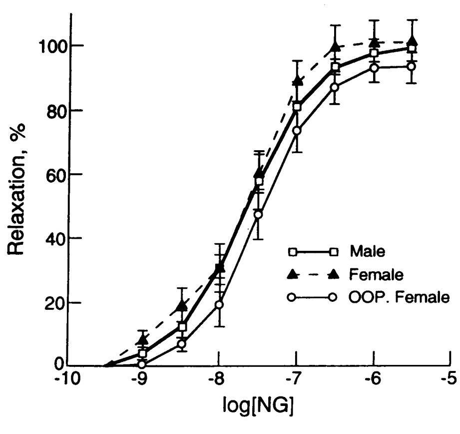

FIG. 4. Mean ± SEM percent relaxation of endothelium-denuded aortic rings from male, female, and oophorectomized (OOP) rabbits to different NG concentrations. Aortic rings were submaximally contracted with phenylephrine before obtaining cumulative responses to NG; responses were similar in all three groups.

aortic rings, no differences in the concentration of either arginine (males, 284.88 ± 82.44 nmol/g of wet weight; females, 410.27 ± 95.15 nmol/g of wet weight; and oophorectomized females, 311.33 ± 92.53 nmol/g of wet weight) or citrulline (males, 43.66 ± 5.21 nmol/g of wet weight; females, 51.88 ± 6.67 nmol/g of wet weight; and oophorectomized females, 46.40 ± 13.30 nmol/g of wet weight) were seen. These values represent means (± SEM) obtained from six to seven animals in each group.

DISCUSSION

The precise role of the unstable endothelium-derived vasodilator NO in the pathogenesis of atherosclerosis is not exactly known. However, considerable evidence suggests that the release of NO from atherosclerotic vessels of rabbits is impaired (21, 22). Recently, in oophorectomized cynomolgus macaque monkeys (*Macaca fascicularis*) fed an atherogenic diet, simultaneous administration of E_2 has been demonstrated to significantly inhibit coronary artery atherosclerosis (23). Furthermore, with quantitative coronary angiography in oophorectomized adult female cynomolgus monkeys whose coronary arteries were rendered atherosclerotic, intracoronary infusion of the endothelium-dependent vasodilator ACh was shown to paradoxically constrict coronary arteries. After E_2 administration, ACh tended to minimally dilate the coronary vessel, and this altered response was not related to plaque extent (24).

Our primary objective here, therefore, was to compare the relative NO release from endothelium-intact aortic rings from male and female rabbits in the basal state and after stimulation by endothelium-dependent vasodilators. We also assessed whether the differences in NO release, if any, could be explained by differences in circulating estrogen concentrations. We selected ACh and the calcium ionophore A-23187 as the endothelium-dependent vasodilators to release NO. ACh was selected as it consistently produces endothelium-dependent relaxation of rabbit aortic rings by a receptor-mediated mechanism; A-23187 was selected as it is an endothelium-dependent vasodilator that is receptor-independent in action. Both ACh and A-23187 are potent releasers of NO (3, 7, 25). In addition to comparing basal (tone related) and stimulated release of NO from endothelium-intact aortic rings, we also wanted to elucidate differences, if any existed, in the responsiveness of endothelium-denuded aortic rings from male and female rabbits to agents that release NO. We elected to use NG, as this is a stable compound that releases NO in vascular smooth muscle (26, 27). Results from these studies would, therefore, indicate whether NO release from endothelial cells and NO actions on vascular smooth muscles show any gender difference.

The basal release of NO was assessed indirectly by initially inducing moderate active tone and then by observing the effects of SOD and L-NMA on changes in basal tone. In the present study, basal release of NO from endothelium-intact aortic rings from female rabbits (ovary intact) significantly increased, when compared with those from males. Addition of SOD to the Krebs bicarbonate in the muscle bath produced more relaxation of aortic rings from female rabbits compared with those from males. This effect was probably due to inhibition of the inactivation of NO released by endothelial cells by the superoxide radical generated in the muscle bath. That the increased relaxation of aortic rings from female rabbits after SOD was, indeed, due to increased NO release, was strengthened by the observation that addition of oxyhemoglobin to the muscle bath abolished the SOD-induced relaxation by inactivating the NO released by the endothelium (28, 29). The increased basal release of NO from endothelium-intact aortic rings of female rabbits when compared with males was further confirmed by observing the effects of adding either L-NMA or L-NAME to the muscle bath. L-arginine is converted to L-citrulline in endothelial cells (30, 31) by the enzyme NO synthase (32), and NO is formed as a byproduct of this reaction. NO synthesis can, therefore, be inhibited by using certain analogs of L-arginine (33, 34). Differences in basal NO formation would, therefore, be reflected in the amount of contraction of the endothelium-intact aortic rings in the presence of arginine analogs. After either L-NMA or L-NAME was added to the muscle bath, the contraction of endothelium-intact aortic rings from female rabbits (ovary intact) was significantly greater in magnitude than that of aortic rings from male rabbits. The contractile effects of these two arginine analogs were readily reversible by adding 3- to 4-fold excess of L-arginine but was not reversed by adding D-arginine, indicating that the effects seen were, indeed, due to increased NO synthesis from L-arginine.

In contrast to the gender differences seen in basal NO release, we observed no differences in the responsiveness of aortic rings from male and female rabbits to endothelium-dependent and endothelium-independent vasodilators. This contrasts with reports by other investigators that chronic treatment of oophorectomized animals with estrogens enhances endothelium-dependent relaxation in some arteries (35–37). These differences may be explained on the basis that supraphysiological concentrations of E_2 were used in those studies, whereas our studies were conducted in animals with normal physiological concentrations of circulating E_2. Furthermore, the ability of E_2 to enhance endothelium-dependent relaxation may vary with the blood vessel studied.

The absence of any gender differences in the responsiveness of endothelium-denuded aortic rings to NG indicates that the observed gender difference in basal NO release is solely due to an increased release of NO from endothelial cells and is not due to increased sensitivity of vascular smooth muscle to the NO released.

The mechanism by which E_2 prevents cardiovascular disease and whether the protective effect is due to a direct action of E_2 on endothelial cells or whether it can be explained on the basis of changes in the serum concentrations of lipoproteins induced by estrogen administration (38, 39) is still unsettled. The gender difference in basal NO release reported in the present study is unlikely to be mediated by circulatory lipids, as total cholesterol and high density lipoprotein cholesterol values were similar in male and female rabbits. On the other hand, the differences seen are most likely mediated by E_2 secreted by the ovaries. Oophorectomy decreased circulating E_2, and basal NO release from aortic rings in this group of animals was significantly attenuated when compared with that seen in ovary-intact females and did not differ from that seen in males. Furthermore, there were no differences in the responses of aortic rings from normal female rabbits and oophorectomized rabbits to endothelium-dependent and endothelium-independent vasodilators, confirming that physiological concentrations of E_2 affect only the basal release of NO but do not modulate the responses of rabbit aortic rings to either endothelium-dependent or endothelium-independent vasodilators.

Arginine concentrations in the aortic rings and in plasma showed no gender differences. Therefore, the gender difference in relation to basal NO release, observed here, cannot be explained on the basis of diminished availability of the substrate for NO synthesis because at concentrations of L-arginine in endothelial cells, conversion of L-arginine to L-citrulline and NO can occur at a maximal velocity compatible with the reported K_m and V_{max} of NO synthase activity in bovine aortic endothelium (32). The observations that only the basal (and not the stimulated) release of NO is increased from endothelium-intact aortic rings from female rabbits, compared with those from males or oophorectomized females, can be explained by a hypothesis suggested by other investigators (40), who speculated that stimulated NO release arises from a pool of endothelial NO separate from the pool associated with basal NO release. E_2 has also been reported (41) to increase prostacyclin (PGI_2)-like activity in the female rat thoracic aorta. Synergy in inhibition of platelet aggregation and adhesion of platelets to endothelial cells has been reported between NO and PGI_2 (42–44). This interaction between NO and PGI_2 stimulated by E_2 may also be important in the prevention and progression of the atherosclerotic process.

Although the precise mechanism by which E_2 increases the basal NO release from endothelium-intact aortic rings has not been addressed here, the differences in release could be from a variety of factors—including increased NO synthase protein, increased activity of the enzyme because of an increase in the concentration of certain cofactors, decreased intracellular inactivation of NO formed either by increased intracellular SOD formation, or by decreased intracellular superoxide anion generation. Delineating these mechanisms is important because this information could contribute to better understanding the modulation of NO synthesis in atherosclerosis.

We acknowledge the excellent technical assistance of Janis Cuevas. The RIAs of E_1 and E_2 were done by Dr. J. K. H. Lu of the Department of Obstetrics and Gynecology, University of California, Los Angeles. This work was partially supported by a grant from the Japan Foundation for Aging and Health, U.S. Public Health Service Grants HL-46843, HL-40872, HL-40922, and HL-35014 and a grant from the Laubisch Fund for Cardiovascular Research.

1. Kannel, W. B., Hjortland, M. C., McNamara, P. M. & Gordon, T. (1976) *Ann. Int. Med.* **85**, 447–452.
2. Gerrity, R. G. (1981) *Am. J. Pathol.* **103**, 181–190.
3. Furchgott, R. R. & Zawadzki, J. V. (1980) *Nature (London)* **288**, 373–376.
4. Palmer, R. M. J., Ferrige, A. G. & Moncada, S. (1987) *Nature (London)* **327**, 524–526.
5. Ignarro, L. J., Buga, G. M., Wood, K. S., Byrns, R. E. & Chaudhuri, G. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 9265–9269.
6. Furchgott, R. F. (1984) *Annu. Rev. Pharmacol. Toxicol.* **24**, 175–197.
7. Gryglewski, R. J., Moncada, S. & Palmer, R. M. J. (1986) *Br. J. Pharmacol.* **87**, 685–694.
8. Bath, P. M. W., Hassall, D. G., Gladwin, A. M., Palmer, R. M. J. & Martin, J. F. (1991) *Arterioscler. Thromb.* **11**, 254–260.
9. Radomski, M. W., Palmer, R. M. J. & Moncada, S. (1987) *Lancet* **ii**, 1057–1058.
10. Galle, J., Busse, R. & Bassenge, E. (1991) *Arterioscler. Thromb.* **11**, 1712–1718.
11. Patthy, A., Basusz, S. & Patthy, L. (1977) *Acta Biochim. Biophys. Acad. Sci. Hung.* **12**, 191–196.
12. Ignarro, L. J., Adams, J. J., Horowitz, P. M. & Woods, K. S. (1986) *J. Biol. Chem.* **261**, 4997–5002.
13. Schweitzer, V. G., Woodson, T. B., Mawhinney, T. D., Rarey, L. E., Bauman, M. J., Raymer, S. L. & Peterson, E. (1990) *Otolaryncol. Head Neck Surg.* **103**, 981–985.
14. Fernstrom, M. H. & Fernstrom, J. D. (1981) *Life Sci.* **29**, 2119–2130.
15. Gold, M. E., Bush, P. A. & Ignarro, L. J. (1989) *Biochem. Biophys. Res. Commun.* **164**, 714–721.
16. Hecker, M., Mitchell, J. A., Harris, H. J., Katsura, M., Thiemermann, C. & Vane, J. E. (1990) *Biochem. Biophys. Res. Commun.* **167**, 1037–1043.
17. Lu, J. K. H., Gilman, D. P., Meldrum, D. R., Judd, H. L. & Sawyer, C. H. (1981) *Endocrinology* **108**, 836–841.
18. Brenner, P. F., Guerrero, R., Cekan, Z. & Diczfalusy, E. (1973) *Steroids* **22**, 774–794.
19. Witztum, J. L., Simmons, D., Steinberg, D., Beltz, W. F., Weinreb, R., Young, S. G., Lester, P., Kelly, N. & Juliano, J. (1989) *Circulation* **79**, 16–28.
20. Lipid Research Clinics Program (1982) *Manual of Laboratory Operations*, U.S. Dept. of Health, Education and Welfare, Publ. No. (NIH) 76-628 (U.S. GPO, Washington), Vol. 1, Ed. 2.
21. Galle, J., Busse, R. & Bassenge, E. (1991) *Arterioscler. Thromb.* **11**, 1712–1718.
22. Forstermann, U., Mugge, A., Acherd, U., Haverich, A. & Frolich, J. C. (1988) *Circ. Res.* **62**, 185–190.
23. Adams, M. R., Kaplan, J. R., Manuch, S. B., Koutnik, D. R., Parks, J. S., Wolfe, M. S. & Clarkson, T. B. (1990) *Arteriosclerosis* **10**, 1051–1057.
24. Williams, J. K., Adams, M. R. & Klopfenstein, S. (1990) *Circulation* **81**, 1680–1687.
25. Peach, M. J., Singer, H. A. & Loeb, A. L. (1985) *Biochem. Pharmacol.* **34**, 1867–1874.
26. Katsuki, S., Arnold, W., Mittal, C. K. & Murad, F. (1977) *J. Cyclic Nucleotide Res.* **3**, 23–35.
27. Ignarro, L. J., Lipton, H., Edwards, J. C., Baricos, W. H., Hyman, A. L., Kadowitz, P. J. & Gruetter, C. A. (1981) *J. Pharmacol. Exp. Ther.* **218**, 739–749.
28. Murad, F., Mittal, C. K., Arnold, W. P., Katsuki, S. & Kimura, H. (1978) *Adv. Cyclic Nucleotide Res.* **9**, 145–158.
29. Martin, W., Villani, G. M., Jothianandan, D. & Furchgott, R. F. (1985) *J. Pharmacol. Exp. Ther.* **232**, 708–716.
30. Palmer, R. M. J., Ashton, D. S. C. & Moncada, S. (1988) *Nature (London)* **333**, 664–666.
31. Sakuma, I., Shiehr, D. J., Gross, S. S., Nathan, C. & Levi, R. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 8664–8667.
32. Forstermann, U., Pollock, J., Schmidt, H. H. H. W., Heller, M. & Murad, F. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 1788–1792.
33. Rees, D. D., Moncada, S. & Palmer, R. M. J. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 3375–3378.
34. Aisika, K., Gross, S. S., Griffith, O. W. & Levi, R. (1989) *Biochem. Biophys. Res. Commun.* **160**, 881–886.
35. Gisclard, V., Miller, V. M. & Vanhoutte, P. M. (1988) *J. Pharmacol. Exp. Ther.* **244**, 19–22.
36. Miller, V. M., Aarhus, L. L. & Vanhoutte, P. M. (1988) in *Proceedings of the Second International Symposium on Resistance Arteries*, eds. Halpern, W., Brayden, J., McLaughlin, M., Oslo, G., Pegham, B. L. & Machey, K. (Perinatology, Ithaca, NY), pp. 136–145.
37. Miller, V. M., Gisclard, V. & Vanhoutte, P. M. (1988) *Phlebology* **3**, 63–69.
38. Stampfer, M. J., Willett, W. C., Colditz, G. A., Rosner, B., Speizer, F. E. & Hennekens, C. H. (1985) *N. Engl. J. Med.* **313**, 1044–1049.
39. Bush, T. L., Barrett-Connor, E., Cowlan, L. D., Criqui, M. H., Wallace, R. B., Suchindran, C. M., Tyroler, H. A. & Rifkind, B. M. (1987) *Circulation* **75**, 1102–1109.
40. Aisaka, K., Gross, S. S., Griffith, O. W. & Levi, R. (1989) *Biochem. Biophys. Res. Commun.* **163**, 710–717.
41. Karpati, L., Chow, F. P. R., Woollard, M. L., Hutton, R. A. & Dandona, P. (1980) *Clin. Sci.* **59**, 369–372.
42. Radomski, M. W., Palmer, R. M. J. & Moncada, S. (1987) *Br. J. Pharmacol.* **92**, 181–187.
43. Radomski, M. W., Palmer, R. M. J. & Moncada, S. (1987) *Br. J. Pharmacol.* **92**, 639–646.
44. Macdonald, P. S., Read, M. A. & Dusting, G. J. (1988) *Thromb. Res.* **49**, 437–449.